11003326-0677

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED OFFICE (DO/US)

PCT/SE00/01380	29 June 2000	30 June 1999
International Application Number	International Filing Date	Priority Date(s) Claimed

MUTATED Nurr1 GENE
Title of Invention

Silvia Buervenich; Lars Olson; Maria Anvret and Andrea Carmine
Applicant(s) for DO/US

"Express Mail" Label No EL608305322US

Date of Deposit **December 28, 2001**

I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the. Assistant Commissioner for Patents, Washington, D C. 20231

Michael Hanes
(Type or print name of person mailing paper or fee)

Michael Hanes
(Signature of person mailing paper or fee)

BOX PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

To the United States Designated Office (DO/US):

I. Accompanying this transmittal letter are certain items which are required under 35 U.S.C. 371 in order that United States National processing of the above identified International application may commence:

( ) at the expiration of the applicable time limit under PCT Articles 22 and 39(1) according to the provisions of 35 U.S.C. 371(b).

(X) as soon as possible upon receipt of this express request under 35 U.S.C. 371(f).

1. The U.S. National fee [35 U.S.C. 371(c)(1)]

a. ( ) was previously transmitted by applicant on (date)____.

b. (X) is submitted herewith as follows:

ENTITY FOR FEE	NO. FILED	NO. EXTRA	SMALL ENTITY RATE	FEE	or	OTHER THAN SMALL RATE
Basic Fee	(USPTO NOT ISA OR IPEA)		////	$520	or /////	**$1,040**
Total Claims	**33**-20 =	13	x 9 =		or x18 =	**$ 234**
Ind. Claims	**5**	2	x 42 =		or x84 =	**$ 168**
(X) Multiple Dependent Claim Presented			+140 =		or +280 =	**$ 280**
	TOTAL NATIONAL FEE		$____		or	**$ 1,722**

i. ( ) Enclosed are two checks which total the amount of to cover the basic filing fee, the multiple dependent claim fee, and the excess independent claim fee.

ii. (X) *Please charge the* filing fee, multiple dependent claim fee (if applicable), excess independent claims fee (if applicable), and *excess total claims fee (if applicable) to Deposit Account No. 23-1703.*

iii. (X) **The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-1703.** A duplicate copy of this sheet is enclosed.

2. A copy of the International application as filed [35 U.S.C. 371(c)(2)]:

a. (X) is transmitted herewith.

b. ( ) is not required as the application was filed with the United States Receiving Office.

c. ( ) has been transmitted

i. ( ) by the International Bureau. Date of mailing of the application (from form PCT/IB/308): _____ A copy of form PCT/IB/308 is enclosed.

ii. ( ) by applicant on (date) _____.

3. A translation of the International application into the English language [35 U.S.C. 371(c)(2)]:

   a. ( ) is transmitted herewith.

   b. (X) is not required as the application was filed in English.

   c. ( ) was previously transmitted by applicant on (date)_____.

4. Amendments to the claims of the International application under PCT Article 19 [35 U.S.C. 371(c)(3)]:

   a. (X) are transmitted herewith - see attached PCT/IPEA/409 dated 8.10.2001

   b. ( ) have been transmitted

      i. ( ) by the International Bureau. Date of mailing of the amendments (from form PCT/IB/308): _____.

      ii. ( ) by applicant on (date) _____.

   c. ( ) have not been transmitted as

      i. ( ) no notification has been received that the International Searching Authority has received the Search Copy.

      ii. ( ) the Search Copy was received by the International Searching Authority but the Search Report has not yet issued. Date of receipt of Search Copy (from form PCT/ISA/202): _____.

      iii. ( ) applicant chose not to make amendments under PCT Article 19. Date of mailing of Search Report (from form PCT/ISA/210): .

      iv. ( ) the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

5. A Translation of the amendments to the claims under PCT Article 19 [35 U.S.C. 371(c)(3)]:

   a. ( ) is transmitted herewith.

   b. (X) is not required as the amendments were made in the English language.

   c. ( ) has not been transmitted for reasons indicated at point I.4.b. or c. above.

6. An original executed declaration for patent application of the inventors [35 U.S.C. 371(c)(4)] complying with 35 U.S.C. 115:

   a. ( ) was previously submitted by applicant on (date) ____________.

   b. ( ) is submitted herewith; and such oath or declaration

      i. ( ) is attached to the application.

      ii. ( ) identify the application and any amendments under PCT Article 19 which were transmitted as stated in points 1.2.b. or c. and 1.4. and states that they were reviewed by the inventor as required by 37 CFR 1.70.

   c. (X) will be submitted subsequently.

II. Concerning other documents:

1. An International Search Report or Declaration under PCT Article 17(2)(a):

   a. ( ) has been transmitted by the International Bureau. Date of mailing (from form PCT/IB/308): _______ A copy of form PCT/IB/308 is enclosed

   b. ( ) is not required as the application was searched by the United States International Searching Authority.

   c. (X) A copy of the International Search Report is transmitted herewith.

   d. ( ) has been submitted by applicant on (date) __________.

2. A Statement of prior art under 37 CFR 1.97 and 1.98:

   a. ( ) is transmitted herewith including copies of the references cited on the attached form PTO-1449.

b. ( ) will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. 371(c).

c. ( ) was previously submitted by applicant on _____, in application serial no. _____.

3. ( ) An original executed Assignment is transmitted herewith for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

a. ( ) **Please charge the $40.00 assignment recordation fee to Deposit Account No. 23-1703.**

b. ( ) Enclosed is a check in the amount of $___.

4. **Other document(s) or information included:**
- Copy of PCT/IPEA/409 - International Preliminary Examination Report;
- 2 Sheets of Drawings
- Return postcard.

Respectfully submitted,

Andrew Fessak

Date: December 28, 2001

Andrew Fessak
Reg. No. 48,528
Applicants' Agent
Attorney's Direct Line (212)-819-8437
Customer No. 007470
(212) 819-8200

Enclosures

MUTATED Nurr1 GENE

Technical field

The present invention relates to novel mutations identified for the first time in the Nurr1 gene.

Background

Clinical conditions associated with defects of specific genes may be inherited or have alternatively been caused by a later developed mutation in the afflicted individual. It is assumed that many neurological and psychiatric conditions have hereditary components, and indeed, schizophrenia, bipolar affective disorder and Parkinson's disease have in common that affected relatives constitute the largest epidemiological risk factor. However, the mode of inheritance of susceptibility is complex and has not yet been elucidated. A further common observation in said three different diseases is etiologic and/or therapeutic involvement of dopaminergic neurotransmission. Thus, cells of the mesostrial midbrain dopamine (DA) neuron system degenerate in Parkinson's disease (Hornykiewicz, O. Biochemical aspects of Parkinson's disease. *Neurology* **51**, S2-S9 (1998)), while the mesolimbic DA axis is the target of several antipsychotic drugs (Gerner, R.H., Post, R.M. & Bunney, W.E.J. A dopaminergic mechanism in mania, *Am J. Psychiatry* **133**, 1177-1180 (1976); and Creese, I., Burt, D.R. & Snyder, S.H. Dopamine receptor binding predicts clinical and pharmacological potencies of antischizophrenic drugs. *Science* **192**, 481-483 (1976)). Recently, a number of studies have pointed out the importance of retinoids and retinoid-related genes for DA cells. Midbrain DA neurons express receptors for retinoic acid-mediated transcription, and mice lacking the retinoid-related receptor Nurr1 fail to develop mesencephalic DA neurons (Zetterström, R.H. *et al.* Dopamine neuron aenesis in Nurr1-deficient mice. *Science* **276**, 248-250 (1997).

The immediate early gene Nurr1 (also called *NOT* in humans, classified as NR4A2 according to the most recent nomenclature [Nuclear Receptors Nomenclature

Committee, 1999]) codes for a nuclear orphan receptor of the NGFI-B family of transcription factors (Gerner et al., *supra*; Hazel, T.G., Nathans, D. & Lau, L.F. A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *Proc Natl Acad. Sci.* USA **85**, 8444-8448 (1988); and Mages, H.W., Rilke, O., Bravo, R., Senger, G, G. & Kroczek, R.A. NOT, a human immediate-early response gene closely related to the steroid/thyroid hormone receptor NAK1/TR3. *Mol. Endocrinol.* **8**, 1583-1591 (1994)). The major splice variant is a 598 amino acid protein with ligand binding, DNA binding and N-terminal domains. Recently, further alternatively spliced mRNA variants encoding the entire N-terminal and DNA binding domains but shorter C-terminal domains have been isolated (Ichinose, H. Et al. Molecular cloning of the human Nurr1 gene: characterization of the human gene and cDNAs. *Gene* **230**, 233-239 (1999); Torii, T., Kawarai, T., Nakamura, S. & Kawakami, H. Organization of the human orphan nuclear receptor Nurr1 gene. *Gene* **230**, 225-232 (1999); and Ohkura, N., Hosono, T., Maruyama, K., Tsukada, T. & Yamaguchi, K. An isoform of Nurr1 functions as a negative inhibitor of the NGFI-B family signaling. *Biochim. Biophys. Acta* **1444**, 69-79 (1999)). While no activating ligand for Nurr1 has been identified to date, it has been shown that Nurr1 can be both constitutively active as a transcription factor and dimerize with RXR, a receptor involved in mediating retinoic-acid induced transcription (Perlmann, T. & Jansson, L. A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and Nurr1. *Genes Dev.* **9**, 769-782 (1995)). These studies together with other observations have made Nurr1 a suggestive candidate gene for diseases with an involvement of the dopamine neuron system, such as schizophrenia, manic depressive illness and Parkinson's disease. However, up to date, no evidence has been provided showing any such involvement, and thus, no effective cures have yet been developed based on such genetic knowledge. As about 1% of the GDP of an industrial country each year is spent on patients suffering from diseases such as schizophrenia and bipolar affective disorder, a clear understanding of the genetic background could hopefully lead to effective treatment schemes and diagnoses. Accordingly, within this field, there is a strong need

based both on human and economical factors of gaining a more thorough understanding.

Previously, certain genes associated with specific, mental conditions have been disclosed. For example, US 5 783 680 discloses monoxidase genes and proteins associated with abnormal behavior. More specifically, the conditions defined therein are behavioural disorders, such as impulsive aggression, and include borderline mental retardation and aggressive outbursts, often in response to anger, fear or frustration. Such aggressive behaviour was noted to vary markedly in severity. Other types of impulsive behaviour that occurred in individual cases included arson, attemted rape, and exhibitionism. Thus, as is obvious to one of skill in this field, there are clear differences between the conditions which US 5 783 680 relates to and phsyciatric disease, such as schizophrenia. This is evident from the fact, that none of the symptoms cited in said US 5 783 680 are found in the DSM-IV diagnostic criteria for schizophrenia or manic depression, nor are the minimal features required for schizophrenia or manic depression present in the family mentioned in US 5 783 680. (For the DSM-IV criteria, see Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition. Copyright 1994 American Psychiatric Association.) Thus, US 5 783 680 does not relate to mutations that can cause psyciatric disturbances.

Finally, Zetterström et al (Science, vol. 276, 11 April, 1997: "Dopamine Neuron Agenesis in Nurr1-Deficient Mice") have shown that mice that lack Nurr1 fail to generate midbrain dopaminergic neurons and suggest Nurr1 ligands as useful for the treatment of Parkinson's disease.

Summary of the invention

For the first time, the present invention provides three mutations of the human Nurr1 gene as defined in the appended claim 1 and further shows the relation thereof with a previously unreported subgroup of ca. 1% of the conditions schizophrenia

and bipolar affective disorder. The novel mutations according to the invention are herein denoted M1, M2 and M3.

Brief description of the drawings

Figure 1 is an overview of the genomic structure of the human Nurr1 gene, distribution of PCR fragments 0-7 and localization of mutations M1-M3 according to the invention in exon 3.

Figure 2 shows autoradiographs of the three heterozygous mutations M1-M3 according to the invention.

Figure 3 shows in table 1 the primers used to amplify the PCR fragments 0 to 7.

Figure 4 provides by table 2 clinical descriptions of the three heterozygous carriers of mutations M1 to M3 according to the invention.

Definitions

In the present application, the term Nurr1 is intended to encompass both mammalian Nurr1 genes, present for example in humans or rodents, and Nurr1 genes of non-mammalian species, such as zebrafish or lamprey. Further, all genes, gene fragments or cDNA species identical to Nurr1 and its cDNA, but published under other names (for example NOT (in humans), RNR1 (in rats) and HZF-3 (in rats)) are also encompassed by the terma Nurr1 as used herein. Thus, any mode of writing such as Nurr1, *Nurr1*, NURR1 and *NURR1* as used herein is to be understood to encompass all of these aspects.

In the present application, the term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and encompasses known analogs of natural nucleotides that can function in a similar manner as naturally occurring nucleotides. When reference is made to the gene, it is to be understood that both DNA and cDNA are encompassed.

The term "variant" as used herein means any functional equivalents of the Nurr1 gene including naturally occurring genes present in other species, and mutants, whether naturally occurring or engineered.

In the present specification, the term "highly stringent conditions" means hybridisation to filter-bound DNA in 0.5M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65^0C and washing in 0.1x SSC/0.1% SDS at 68^0C (Ausubel F. M. *et al.*, eds., 1989, *Current Protocols in Molecular Biology*, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3). In instances wherein the nucleic acid molecules are deoxyoligonucleotides ("oligos"), highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37^0C (for 14-base oligos), 48^0C (for 17-base oligos), 55^0C (for 20-base oligos), and 60^0C (for 23-base oligos). Low stringency conditions are well known to those of skill in the art, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook *et al.*, 1989, *Molecular Cloning, A Laboratory Manual*, Cold Springs Harbor Press, N.Y.; and Ausubel *et al.*, 1989, *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley, Interscience, N. Y.

Detailed description of the invention

Thus, in a first aspect, the present invention relates to an isolated Nurr1 gene including one or more mutations selected from the group consisting of Met97Val M97V), His103Arg (H103R), Tyr121del (Y121del) and Tyr122 (Y122del), or a functional fragment or variant thereof. The fragments encompassed by the present invention may comprise one or more of the present mutations surrounded by wild type sequence or adjacent to wild type sequence on one side thereof. In this context, reference is made to Figure 1 showing an overview of the genomic structure of Nurr1 and the mutations according to the invention. The human Nurr1 gene is comprised of 8 kb as 8 exons and 7 introns located on chromosome 2q22-23, see Ichino-

se et al., *Gene* **230** (1999) 133-239, which gene has been deposited in the DDBJ/EMBL/GenBank with accession number AB017586. (For the entire sequence of mouse Nurr1, see Castillo *et al., Genomics* 41, 250-257 (1997), Fig 1, accession no. U86783.) In one embodiment, a fragment according to the invention comprises all the exons of the Nurr1 gene, *i.e* all of the cDNA sequence thereof. More specifically, as the herein defined novel mutations are present on exon 3, in one embodiment, a fragment according to the invention comprises at least exon 3 of Nurr1. Specific embodiments of the first aspect are fragments comprising the mutation Met97Val, wherein a G in the first position of codon no 97 has substituted an A in said position, the mutation His103Arg, wherein a G in the first position of codon no 103 has substituted an A in said position, or the mutation Tyr121del or Tyr122del, wherein a tyrosine has been deleted at position 121 or 122. As easily appreciated by the skilled in this field, and as further discussed in relation to figure 2, the last mentioned deletions are impossible to differentiate from each other, and therefore they will be denoted Tyr122 herein. The present fragments may include any suitable length of the Nurr1 gene depending on the intended use. Further, in the present context, it is noted that the present invention relates to mutated Nurr1 genes of human and mouse origin, irrespective of whether it is written in capital letters or not. Various advantageous uses of the present genes and fragments will be discussed in further detail below.

Accordingly, by disclosing the novel mutations defined above, the present invention shows for the first time a linkage between the Nurr1 gene and neuro-psychiatric disorders, which linkage has previously been sought for without any success, see e.g. Ichinose *et al., supra*. Previously, when such a possible link was discussed, a general connection to neuro-psychiatric conditions was suggested but never substantiated. However, it has never been proposed or foreseen that such a link would exist only to a specific selection of said conditions. Accordingly, the evidence presented herein that mutations in the Nurr1 gene are linked to schizophrenia and manic depressive illness, but not to Parkinson's disease, is highly surprising and will enable

an essential step forward in studies aimed at developing new drugs as well as new diagnostic tools within this field. Furthermore, the invention is of importance for the diagnostic field of schizophrenia and bipolar affective disorder because the finding of Nurr1 mutations in a subgroup of patients will help to better characterize the ill-defined and inhomogenous group of patients suffering from these conditions. Thus, those patients suffering from schizophrenia or bipolar affective disorder because of mutated Nurr1 can now be identified and treated differently and more specifically according to their specific genetic defect, which has been revealed for the first time in the present invention.

Thus, even though Zetterström et al (Science, 1997, supra) suggested that putative Nurr1 ligands could be useful for the treatment of Parkinson's disease and other disorders of midbrain dopamine circuitry, they could not show any link between Nurr1 and the herein discussed conditions of schizophrenia and manic depression. The present invention discloses for the first time three specific mutations which as shown below in the experimental section are clearly linked to a subgroup of patients suffering from said conditions. Although the three disclosed mutations cannot explain the condition in all schizophrenia or manic depressed patients, their absence from investigated control material strengthens their role in the patients where they have been identified. Since an effect of each one of the mutations on Nurr1 activity has been shown, and all three mutations show very similar effects, reliable evidence is provided that the present mutations contribute to the phenotype observed in those patients in which mutations were found.

To further stress the practical usefulness of the present invention, it is noted that a person skilled in the art is well aware of the multifactorial etiology of complex diseases, such as schizophrenia and manic depression. It is therefore unlikely that one would find mutations in a single gene in a large portion of patients. Given a worldwide prevalence of both diseases of about 1% each, explaining 1% of these diseases can help an enormous number of people.

Actually, after above discussed description of mutations in MAOA in patients with impulsive aggression (US 5 783 680), another study (Am J Med Genet 1999 Feb 5;88(1):25-8)was carried out searching for MAOA mutations in patients with similar diagnoses (and also healthy individuals and Parkinson patients who served as control material), without finding any muations. Thus, similarly to the novel mutations according to the present invention, MAOA mutations are very rare causes of certain conditions, which in their case are behavioural.

Another aspect of the present invention is a nucleic acid capable of specifically hybridising to a gene or a fragment as defined above. The preparation of DNA is easily accomplished by the skilled in this field by any suitable method, such as cloning and restriction of appropriate sequences by any *in vitro* or *in vivo* method, such as the polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS), the self-sustained sequence replication system (SSR) or cell based cloning and amplification, wherein the cell may be any suitable cell, such as a bacterium, a cultured cell line etc. A wide variety of cloning and amplification methods are well known to the skilled in this field, see e.g. Sambrook *et al.*, (1989); *Molecular Cloning: A Laboratory Manual*, 2nd Ed, Vol. 1-3). Further, they may be prepared by direct chemical synthesis by methods such as the phosphotriester method (Narang *et al.*, *Meth. Enzymol.* **68**, 90-99 (1979); the phosphodiester method (Brown *et al.*, *Meth. Enzymol.* **68**, 109-151 (1979); the diethylphosphoramidite method (Beaucage *et al.*, *Tetra. Lett.*, **22**, 1859-1862 (1981); and the solid support method (US patent no. 4 458 066).

The present fragments may e.g. be used as primers, e.g. for PCR, which for example may be present in a kit, as probes etc. Further, as discussed below, they may be used to generate immunogenic polypeptides or fusion proteins for use in generating specific antibodies which recognise the mutant epitope. For use as primers, the frag-

ments are preferably of a length of at least about 10, such as at least about 15-20, bases, which however depends on the method used.

A second aspect of the invention is a vector comprising a nucleic acid according to the invention as well as to cells harbouring such a nucleic acid useful for the production of the present protein or peptide. Such cells may be procaryotic or eucaryotic and are described in more detail below in relation to cell culture model systems.

A third aspect of the invention is a protein or a peptide encoded by a gene or a gene fragment according to the invention. Accordingly, in specific embodiments, the present protein or polypeptide may include a Val residue in the position corresponding to amino acid no. 97 of the wild type Nurr1 protein; an Arg residue in the position corresponding to amino acid no. 103 of the wild type Nurr1 protein; and/or a sequence that does not include any Tyr residue in the position corresponding to amino acid no. 122 of the wild type Nurr1 protein.

A fourth aspect of the invention is a screening method, wherein a protein or a peptide according to the invention is used as a lead compound to identify substances capable of altering, e.g. inhibiting, partially or totally, suppressing, enhancing, restoring etc, the biological effect of said protein or peptide. Accordingly, the substances identified by the present method will be useful as medicaments for the treatment of psychotic conditions, specifically schizophrenia and/or manic depressive disorder and such substances are also encompassed by the present invention. Methods of screening, such as high throughput screening etc, are known to the skilled in this field, see e.g. US patent nos 5 763 263 and 5 710 266. In brief, such a method may e.g. include

(i) immobilization of the present protein or peptide on a solid support;

(ii) exposure to a library of candidate substances; and

(iii) detecting the binding of protein or peptide to candidate.

Further, the present protein or polypeptide may also be used to specifically design a substance binding thereto and capable of inhibition or other alteration of the function thereof.

Thus, in a fifth aspect, the invention also relates to methods of treatment as well as to the use of the present proteins or peptides in the manufacture of a medicament. Further, the present proteins or peptides are also useful in diagnosis of schizophrenia and/or manic depressive disease, as the expression thereof in a patient will be an indication of the existance of one of the mutations according to the invention.

A further aspect of the invention is an antibody which specifically binds to a protein or peptide according to the invention and preferably comprises at least about 10, more preferably at least 20, 40 or 50 or more amino acids. Such an antibody may be used as a medicament in order to inhibit or suppress the biological effect of the product of a mutated gene. Alternatively, the present antibody may be used in the diagnosis of schizophrenia and/or manic depressive disorder. The present antibody may be polyclonal or monoclonal, preferably, it is a monoclonal antibody. It can be humanized or human. The invention also relates to a cell, such as a recombinant cell, e.g. hybridomas or triomas, expressing such an antibody as defined above. Methods of producing polyclonal antibodies are known to those of skill in the art. In brief, an immunogen (antigen), preferably a purified polypeptide, a polypeptide coupled to an appropriate carrier (*e.g.*, GST, keyhole limpet hemanocyanin, *etc.*), or a polypeptide incorporated into an immunization vector such as a recombinant vaccinia virus (*see,* U.S. Patent No. 4,722,848) is mixed with an adjuvant and animals are immunized with the mixture. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the polypeptide of interest. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the polypeptide is performed where desired (*see, e.g.,* Coligan (1991) *Current Protocols in Im-*

munology Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY).

Monoclonal antibodies are prepared from cells secreting the desired antibody. These antibodies are screened for binding to normal or modified polypeptides, or screened for agonistic or antagonistic activity. Specific monoclonal and polyclonal antibodies will usually bind with a K_D of at least about 0.1 mM, more usually at least about 50 μM, and most preferably at least about 1 μM or better. In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, *etc.* Description of techniques for preparing such monoclonal antibodies are found in, *e.g.* Stites *et al.* (eds) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therin; Harlow and Lane, *supra*; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) *Nature* 256: 495-497.

The pharmaceutical preparations comprising a substance identified by use of the herein described methods according to the invention, or an antibody as disclosed above, according to the invention may be prepared by any standard method according to standard methods, see e.g. *Remington's Pharmaceutical Sciences*, 16th ed. Osol, A (ed.) 1980; and Langer, *Science* **249**, 1527-1533 (1990).

The peptides, polypeptides and proteins according to the invention may be synthesized using standard chemical peptide synthesis techniques well known to the skilled person. For solid phase synthesis, see e.g. Barany and Merrifield, *Solid-Phase Peptide Synthesis*, in *The Peptides: Analysis, Synthesis, Biology*, vol 2*: Special Methods in Peptide Synthesis*.

The present peptides and polypeptides may also be produced using recombinant DNA technology. Generally, this involves creating a DNA sequence that encodes

the desired peptide, i.e., including one or more of the present mutations, e.g. as a recombinant sequence encoding the present peptide as part of a fusion protein, placing the DNA in an expression cassette under the control of a particular promoter, expressing the peptide in a host and isolating the expressed peptide. In case of a protein, the renaturation thereof may be required. The nucleic acids encoding the peptides or proteins may be expressed in a variety of host cells, including E.coli, other bacterial hosts, yeast, and various higher eucaryotic cells, such as COS, CHO and HeLa cell lines and myeloma cell lines. The gene will be operably linked to appropriate expression control sequences for each host. For E.coli, this includes a promoter such as the T7, trp or lambda promoters, a ribosome binding site and preferably a transcription termination signal. For eucaryotic cells, the control sequences will include a promoter and preferably an enhancer and it may include splice donor and acceptor sequences. The expression systems that may be used for the preparation of the present proteins and peptides include but are not limited to microorganisms such as bacteria (e.g., E. coli, B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the present nucleotide sequences; yeast (e.g., Saccharomyces, Pichia) transformed with recombinant yeast expression vectors containing the the present nucleotide sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the the present sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the present nucleotide sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

Once expressed, standard procedures may be used for purification, such as ammonium sulphate precipitation, affinity columns, column chromatography, gel elec-

trophoresis and the like. See, generally, R. Scopes, *Protein Purification*, Springer-Verlag, N.Y. (1982), Deutscher, *Methods in Enzymology*, Vol 182: *Guide to protein purification*, Academic Press, Inc., N.Y. (1990).

A further aspect of the invention is a cell culture comprised of transgenic cells harbouring one or more of the present mutations for use as a model, e.g. for testing novel pharmaceutically active substances. Such cells may carry one or more of the present mutations on a vector or in the genome. In one embodiment, they are isolated cells that have been mutated spontaneously in a natural environment, while another embodiment relates to cells produced by recombinant DNA techniques. Such a cell culture will comprise immortalized cells, such as primary nerve cells, COS or HeLa cells etc. Any one of the various methods currently used for the introduction into and amplification of foreign (heterologous) nucleic acid into cultured cells for research purposes as well as for the purpose of overproduction of proteins of interest for pharmaceutical applications may be used according to the invention. The present cell lines comprise inserted genes or gene fragments as described above. Alternatively, they are derived from a transgenic animal (see e.g. Babinet *et al.*, US patent no. 5 073 490). Transgenic animals are disclosed in further detail below, but a general review of the basic process for the creation of transgenic animals, particularly mice, is found e.g. in Palmiter *et al.*, in "Dramatic Growth of Mice That Develop From Eggs Microinjected with Metallothionein-Growth Hormone Fusion Genes", *Nature*. Vol. **300**, 611-615 (Dec. 1982).

However, mammalian systems are the most preferred. In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome will result in a recombinant virus that is viable and ca-

pable of expressing the present gene product in infected hosts. (See e.g. Logan & Shenk, 1984, *Proc. Natl. Acad. Sci.* USA **81**:3655-3659). Specific initiation signals may also be required for efficient translation of the present inserted nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire Nurr1 gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (See Bittner *et al.*, 1987, *Methods in Enzymol.* **153**, 516-544). Further, a purification of the present product is needed. For example, an antibody specific for the expressed peptide or protein may be used. For example, a system described by Janknecht *et al.* allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, *et al.*, 1991, *Proc. Natl. Acad. Sci.* USA **88**, 8972-8976).

Further, the present invention relates to a transgenic animal, preferably a non-human animal, such as a mammalian, e.g a mouse, comprising a mutated gene or a gene fragment according to the invention, as well as to any further generation of said animal. For a comprehensive instruction on how animal models can be established for human diseases with identified genetic mutations, reference is made to Human Molecular Genetics, BIOS Scientific Publishers Limited, 1996, chapter 19, pp. 507-550, entitled "Studying human gene structure and function and creating animal models of disease", Strachan, T. and Read, A.P. Any technique known in the art may be used to introduce the present gene comprising one or more of the novel mutations into animals to produce the founder lines of transgenic animals. Such techniques

include, but are not limited to pronuclear microinjection (Hoppe, P.C. and Wagner, T. E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten *et al.,* 1985, *Proc. Natl. Acad. Sci.*, USA **82**:6148-6152); gene targeting in embryonic stem cells (Thompson *et al.*, 1989, *Cell* **56**:313-321); electroporation of embryos (Lo, 1983, *Mol Cell. Biol.* 3:1803-1814); and sperm-mediated gene transfer (Lavitrano *et al.*, 1989, *Cell* **57**:717-723); etc. For a review of such techniques, see Gordon, 1989, Transgenic Animals, *Intl. Rev. Cytol.* **115**:171-229. Most preferably, the transgenic animal according to the invention is a mouse comprising one or more of the present mutations M1-M3.

Alternatively, instead of introducing a complete gene or gene fragment into the host, the desired mutation(s) is provoked in a laboratory animal, such as a mouse. This may be accomplished by any mutagenic process, including in vitro-mutagenesis followed by transfer of genomic material into embryonic stem cells or any other mutagenic process, including chemical, biological, radioactive, or ultraviolet treatments.

Due to the fact that the domains of the Nurr1 gene surrounding M1, M2 and M3 are highly conserved in mouse and human, see e.g. Ichinose *et al.*, *supra*, the most preferred transgenic animal model according to the invention is a mouse, wherein one or more of the present mutations M1-M3 have been introduced. Thus, for reasons of simplicity in the preparation, the mouse model is an ideal candidate for studies of the present human mutations.

Methods of transferring genes into the germline, the expression of natural and hybrid genes and phenotypic changes that have occurred in transgenic mice are described by Palmiter and Brinster in *Ann. Rev. Genet.* **20** (1986) 465-499.

Accordingly, the present invention also relates to the use of an animal model as defined above for the identification of new drugs. More specifically, such a method of

testing the therapeutic activity of a pharmacological agent on schizophrenia or manic depressive illness comprises administering an effective amount of said pharmacological agent to the mouse model according to the invention and evaluating said agent's effect on said mouse. Any substance identified by use of a model according to the invention is also within the scope of the invention.

Another aspect of the invention is a method of detecting the presence of a mutation in exon 3 of the Nurr1 gene, which mutation is selected from the group consisting of Met97Val, His103Arg, Tyr121del and Tyr122del, which method comprises obtaining a biological sample from e.g. a human subject or an animal model and analyzing said sample for said mutation. More specifically, the biological sample is analyzed by isolating DNA from said sample, amplifying said DNA, hybridising said DNA to a labeled oligonucleotide probe that specifically hybridizes to mutant DNA containing a G as the first base of codon no 97; a G as the second base of codon no 103; or (how do I say this:) altered TACTAC in codon no 121 or 122, or to one or more bases adjacent to said mutation, depending on the method used. Methods of detecting mutations in DNA are e.g. reviewed in U. Landgren, GATA 9, 1992, pp. 3-8.

Further, the present mutations in the Nurr1 gene can be detected by either of the following methods (a)-(j), which are described in detail in: (1) *Mutation Detection, A Practical Approach*, R.G.H. Cotton, E. Edkins and S. Forrest (editors), The Practical Approach Series, Oxford University press (1998) and (2) *Finding Mutations, The Basics,* J.R. Hawkins, Oxford University Press (1997).

(a) Single strand conformation polymorphism analysis (SSCA, also called SSCP), alone or in combination with heteroduplex analysis (HA) is one of the most widely used approaches for mutation detection. (b) Denaturing gradient gel electrophoresis (DGGE) is the name of a whole family of similar methods that are based on the reduction in electrophoretic mobility of a DNA molecule in a dense medium during

denaturation. (c) The ribonuclease protection assay or RNase cleavage assay (RPA), the chemical cleavage of mismatch (CMM) assay and mutation detection using T4 endonuclease VII (EMC) are effective methods to detect DNA-DNA or DNA-RNA mismatches caused by mutations. (d) Hybridisation with sequence specific oligonucleotide probes (SSOP) takes advantage of the fact that under stringent conditions, a single basepair mismatch can prevent hybridisation of short complementary oligonucleotide probes. (e) Ligation assays such as the oligonucleotide ligation assay (OLA) comprise further ways of detecting changes in DNA sequences. (f) Direct sequencing, which constitutes one of the most sensitive methods of mutation detection. (g) A rather new method originating from direct sequencing is called minisequencing or solid phase minisequencing. (h) Selective amplication of specific alleles (PASA, also called ASA or ASP) makes it possible to detect mutations already during amplification of the fragment of interest by PCR. (i) With the protein truncation test (PTT), one can identify mutations that induce stop codons in DNA sequence after translation of the sequence into protein. (j) Restriction fragment length polymorphism (RFLP) analysis makes use of endonucleases which recognize and cleave specific nucleotide sequences. Mutations in DNA can either change the sequence so that an endonuclease which cuts the wildtype sequence cannot recognize and cut the mutated sequence, or it can induce a new cleavage site for an endonulease not cutting the wildtype sequence. In sequences that cannot be distinguished by naturally occurring enzymes, mutations can be analyzed by primer-introduced restriction analysis, a method which alters the sequence surrounding the mutation.

In addition, the invention also relates to a kit for performing any one of the methods described above. Accordingly, essential agents for mutation detection according to the respective method can be combined in single packages as user-friendly kits for easy mutation detection. Such kits may contain reagents and enzymes for amplification of the mutated sites, oligonucleotides hybridizing to DNA sequences around and/or containing the mutations and enzymes for specific cleavage of DNA at mutated sites. Further, the present kits may also include suitable labels. The present in-

vention also relates to the production of any such kit aimed at detecting the mutations M1-M3 identified by the inventors.

Finally, the present invention also relates to methods of gene therapy aimed at correcting one or more of the mutations according to the invention. Techniques for gene therapy are well known to the skilled artisan. For example, WO 93/24640 and WO 95/11984 disclose methods and compositions for *in vivo* gene therapy using nonviral or viral vector technology. In another example, WO 95/06743 discloses a method whereby therapeutic nucleic acid constructs are introduced into a patient's isolated airway epithelial cells via transformation with a viral vector containing a construct. The transformed cells are then administered to the patient. The technology for the various nonviral or viral vectors, that can be applied to cells *in vitro* or injected or applied *in vivo* to patients are likewise well known to the skilled artisan.

Detailed description of the drawings

Figure 1 is an overview of the genomic structure of the human Nurr1 gene, distribution of PCR fragments 0-7 and localization of the mutations M1-M3 according to the invention in exon 3. All three mutations are localized within 78 basepairs of PCR fragment 1 comprising the first 495 bases of exon 3. Fragment 0 was only sequenced once in order to design a new primer within the second intron closely adjacent to exon 3. All other fragments (1-7) were used for the sequencing of the coding region in the first 20 Parkinson patients, 20 schizophrenics and 4 controls. After identification of the first mutation, only fragment 1 was sequenced in all further patients.

Figure 2 shows autoradiographs of the three heterozygous mutations M1-M3 according to the invention. *a*, M1 (M97V) leads to a double band at the first nucleotide of the codon for amino acid position 97. The arrow points at the wildtype band. *b*, M2 (H103R) exchanges the middle nucleotide in the codon for amino acid 103. The arrow indicates the wildtype. *c*, M3 (ΔY122) deletes one of the tyrosines at positions 121 or 122. It is not possible to tell which three consecutive bands have been dele-

ted. However, the pattern of the 3 basepair shift beginning at the TACTAC sequence must result from a 3 basepair deletion within these six basepairs and the surrounding amino acid sequence is not affected by this shift. Because a reverse primer was used for generating this autoradiograph, the shift occurs in the adjacent sequence above the double tyrosine codons. On the right, the wildtype (wt) sequence is shown for comparison.

Figure 3 shows in table 1 the primers used to amplify the PCR fragments 0 to 7. Because no intronic sequences were known when the present study was initiated, primers are located in exons and almost all fragments contain intron sequence. More specifically, fragment no. 0 comprises a part of the second (non-coding) exon, intron 1 (complete) and 2 bases of exon 3; in fragment no. 1, the forward primer is located in the second intron, immediately adjacent to the border to the third exon, and this fragment contains the first 513 bases of exon 3; fragment no. 2 is the second half of exon 3, both primers being located within coding sequence; fragment no. 3 is the rest of exon 3, complete intron 3 and major part of exon 4; fragment no.4 comprises exon 4, intron 4 and exon 5; fragment no.5 comprises exon 5, intron 5 and exon 6; fragment no.6 comprises exon 6, intron 6 and exon 7; and fragment no.7 comprises exon 7, intron 7 and exon 8 including 34 bases of 3' untranslated region.

Figure 4 provides by table 2 clinical descriptions of the three heterozygous carriers of mutations M1 to M3 according to the invention. In table 2, the following abbreviations are used: MD: Major depression; SAP: Separation Anxiety Disorder; ADHD: Attention Deficit/Hyperactivity Disorder. More specifically, the history of the female heterozygous carrier of M1 is an onset with auditary hallucinations, later displayed delusions of reference, paranoid delusions, verbal auditory hallucinosis, visual and tactile hallucinosis, thought insertion and thought "broadcast", as well as flat and sometimes inappropriate affects. She responded well to antipsychotic treatment but experienced relapses after discontinuation of the antipsychotic medication. She is currently on continuous antipsychotic medication and has been free from psychotic episodes for several years. Her family history was as follows: The paternal grandmother's sister had been treated at a mental hospital, the cause of illness

unknow. The history of the M2 carrier includes a first hospital admission after 5 months of expansive and irritable mood, decreased need for sleep, distractability, and excessive involvement in pleasurable activities. The end of this period also included psychotic symptoms: verbal mood-congruent auditory hallucinations, delusions of reference, grandiose delusions, incoherence, and disorganized behaviour. Treatment was performed with antipsychotic medication and discharge to an outpatient department. Rehospitalization took place a month later due to depressed mood, weight gain, loss of energy, feeling of worthlessness, difficulties to concentrate, and recurrent thought of death. No psychotic symptoms occurred during later manic and depressive episodes. She has currently been treated with lithium for more than four years without relapses. The family history of the M2 carrying patient was as follows: The patient reported no major psychiatric disturbances in the family history. However, the paternal grandfather was said to be a confidence trickster. The history of the M3 carrier includes two episodes of extended depressed mood and anhedonia (ages 6-7 and 10-11). Auditory hallucinations were experienced since the second episode. Hallucinations and delusions occur regularly and are independent of mood state. Intermittent history of obsessions and compulsions since age of 6. Frequent episodes of illogical thinking and neologisms and short periods of incoherent speech. The family history of the M3 carrying patient was as follows: The mother reported a few occasional (olfactory, visual) hallucinatory episodes but no further symptoms.

Experimental

The examples below are given merely to illustrate the present invention and are not intended to limit the scope of the invention as defined by the appended claims. All references given below or elsewhere in the present specification are included herein by reference.

Example 1: Identification of mutations

Methods

General

By direct sequencing of genomic DNA, one missense mutation and one deletion of three basepairs in the third exon of Nurr1 were found in two schizophrenic patients. One missense mutation was found in the same exon in an individual with manic depressive disorder. None of these mutations were present in patients with Parkinson's disease or control DNA material of normal populations.

The entire coding region of the Nurr1 gene was first sequenced in 20 patients with Parkinson's disease, 20 patients with schizophrenia and 4 healthy control individuals. The primers used for PCR amplification of the DNA fragments shown in figure 1 are summarized in table 1. One deletion of three basepairs (ΔY122, fig 1 and 2) was identified in one childhood-onset schizophrenic individual originating from the USA. This mutation (M3) was located in fragment 1 which covers about the first half of the N-terminal domain. This mutation was not found when fragment 1 was sequenced in 100 healthy individuals from the USA, nor were any other mutations found in the same fragment in this material. As mutations are frequently clustered in coding regions of disease-causing genes, the present sequencing efforts were continued by focusing on fragment 1. This fragment was sequenced in 60 Swedish controls, 50 additional Swedish Parkinson patients, 17 additional American schizophrenics, 135 additional Swedish schizophrenics and 29 manic depressed patients from the USA and Sweden. One missense mutation (H103R; M2 in the figures) was identified in a Swedish schizophrenic patient and another missense mutation (M97V; M1 in the figures) was identified in a Swedish patient with manic-depressive illness with psychotic symptoms. All three mutations were absent from all other DNA samples and are therefore unlikely to be polymorphic variants of the human Nurr1 gene. Notably, no mutations were found in the Parkinson DNA samples. Attempts to obtain DNA samples from relatives of the three mutation carriers were only moderately successful. Only in the childhood onset case (M3) was it

possible to obtain parental DNA. The mother was a healthy carrier of the mutation, but no further family history could be obtained in this kindred. The medical and family histories of all three patients are summarized in relation to table 2, figure 4. Interestingly, all three mutations cluster in evolutionary conserved amino acids in close vicinity to each other in the N-terminal region of Nurr1, which might indicate a common effect on Nurr1 function. Preliminary results from *in vitro* mutagenesis experiments indicate that transcriptional activity mediated by Nurr1 monomer binding to the Nurr1 binding response element is not severely affected by any of the three mutations (data not shown). More complex assays measuring Nurr1 heterodimerization with RXR, Nurr1 homodimerization and interaction of Nurr1 with other activating or silencing factors are needed to elucidate possible functional consequences of the mutations. Because the Nurr1 gene is evolutionary highly conserved and all amino acids affected by the mutations are identical in mice, generation of transgenic mice carrying the novel mutations according to the invention provides additional opportunities to understand how Nurr1 function is affected. The generation of such mice will help elucidating one pathway for the development of schizophrenia and/or bipolar affective disorder and thus will also help to find further genetic and environmental etiological factors for these diseases.

Patients

All schizophrenic and bipolar affective disorder patients fullfilled the DSM-III-R (Diagnostic and Statistical Manual of Mental Disorders, Third Revised Edition. Copyright 1987 American Psychiatric Association) or DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition. Copyright 1994 American Psychiatric Association) diagnostic criteria. Parkinson patients were diagnosed according to the "Brain bank clinical criteria" for idiopathic Parkinson's disease (Daniel, S.E. & Lees, A.J. Parkinson's Disease Society Brain Bank, London: overview and research. *J. Neural Transm. Suppl.* **39**, 165-172 (1993)) except that three cases had more than one affected relative but were still included. DNA samples from bipolar affective disorder patients were purchased from the Coriell Cell Repository (Cam-

den, USA). While all schizophrenic and Parkinson patients involved in this study were unrelated individuals, patients with bipolar affective disorder belonged to the Coriell families number 811 (n=3), 812 (n=2), 823 (n=5), 830 (n=4), 834 (n=1) 835 (n=1), 884 (n=5), 888 (n=1), 893 (n=2) and 1075 (n=2). Three manic depressed patients from Sweden were also included in the material as presumed controls or schizophrenic patients. At the present study they turned out to fulfill the DSM-III-R criteria for bipolar affective disorder.

DNA sequencing

DNA was extracted from whole blood accoring to standard protocols. The genomic structure of the human Nurr1 gene was deduced from the homologous mouse Nurr1 gene, and primers covering the second exon and the entire coding region were designed (table 1, fig 3). The numbering of the nucleotides and amino acids employed herein follows the initial publication of the human Nurr1 *(NOT)* mRNA sequence (Mages *et al.*, *supra*). The sequence of the second intron was determined by sequencing of fragment 0, and one additional forward primer was designed located in the second intron (see table 1, figure 3, and figure 1). Polymerase chain reaction (PCR) was carried out using Taq DNA polymerase (SIGMA). 35 cycles were run at 94^0C for 40 seconds, 56^0C for 45 seconds and 72^0C for one minute. After PCR, the samples underwent electrophoresis on 1% low-melting agarose gels and were visualized using UV-translumination after ethidiumbromide staining. DNA was extracted from gel slices (PCR preps DNA purification kit, SDS) and DNA fragments were sequenced (Thermo Sequenase radiolabeled terminator cycle sequencing kit, Amersham). The reaction products were run on 6% acrylamid sequencing gels (National Diagnostics) and visualized by autoradiography.

Results

The present invention shows that two out of 187 patients with schizophrenia and one out of 29 patients with bipolar affective disorder carried unique mutations clustered in the area of the N-terminal domain of the Nurr1 gene. Even though the fre-

quency of mutated Nurr1 determined according to the invention may appear low (about one percent for schizophrenia and three percent for bipolar disorder), it is perfectly in line with the multifactorial origin of these diseases, with a number of different gene defects remaining to be identified explaining a fraction of the total number of patients each.

Example 2: Function of identified mutations

Because the rarity of molecular variants of NURR1 does not exclude their role in the pathogenesis of psychiatric disease in those individuals where they were identified, functional assays were set up in order to elucidate if any of the three clustered mutations might affect *NURR1* function. The following description of results from such functional studies is adapted from the manuscript entitled "*NURR1* Mutations in Cases of Schizophrenia and Manic Depressive Disorder" by authors Silvia Buervenich, Andrea Carmine, Mariette Arvidsson, Fengqing Xiang, Zhiping Zhang, Olof Sydow, Erik G. Jönsson, Göran C. Sedvall, Sherry Leonard, Randal G. Ross, Robert Freedman, Kodavali V. Chowdari, Vishwajit L. Nimgaonkar, Thomas Perlmann, Maria Anvret, and Lars Olson. The cited work is currently in press in the American Journal of Medical Genetics, Neuropsychiatry Section.

In-vitro expression assay

Human *NURR1* cDNA sequence was cloned into the expression vector pCMX [Umesono et al., 1991], and expression vectors for the mutants were generated by site-directed mutagenesis (GeneEditor In Vitro Site-Directed Mutagenesis System, Promega). A double stranded NurRE [Philips et al., 1997] DNA fragment was generated by annealing the primers 5′-AGC TTG TGA TAT TTA CCT CCA AAT GCC AG-3′ and 5′-AGC TCT GGC ATT TGG AGG TAA ATA TCA CA-3′. A luciferase reporter plasmid containing three tandem NurRE sites was generated by ligating the annealed fragments upstream of the herpes simplex thymidine kinase promoter

fused to the luciferase gene. Human embryonic kidney (HEK)-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). Transfections were performed in 24-well plates by the calcium phosphate method. Briefly, cells were seeded one day prior to transfection. Cells in each well were treated with 100ng of the indicated expression vectors, 100ng of reporter plasmid and 200ng of reference CMX-βgal plasmid containing the β-galactosidase gene and CMX-PL1 as carrier DNA up to 500ng of total DNA. Cells were exposed to calcium phosphate precipitate for 12-16h and washed with PBS and then fresh medium was added. The cells were harvested and lysed after 36h incubation. Extracts were assayed for luciferase and β-galactosidase activity in a microplate luminometer/photometer reader (Lucy-1, Anthos). All luciferase activities were normalized to β-galactosidase activity.

NURR1 transcriptional activity was measured using human embryonic kidney (HEK)-293 cells monitoring NURR1 homodimer binding to NurRE [Philips et al., 1997]. Each experiment was carried out using two independent clones for the wild-type and mutated vectors, respectively, and activity was measured in at least four separate wells for each clone. A significant reduction (30-40%) of transcriptional activity of mutated NURR1 homodimers was found. This reduction was strikingly similar in all three mutated clones and is consistent with the present finding that the three mutations are clustered in a region of NURR1 which is critically important for transcriptional activation. The avarage activity and s.e.m. of mutated NURR1 in percent of wildtype activity in all experiments carried out were ΔY122: 0.645 ± 0.024, n=87; H103R: 0.608 ± 0.036, n=87; and M97V: 0.661 ± 0.046, n=54.

The facts that (1) the three mutations were clustered within 78 basepairs of the N-terminal domain, (2) they all lead to impaired NURR1 transcriptional activity in an *in-vitro* bioassay, and (3) except for the three mutations this region of the *NURR1* gene was totally conserved at the DNA level in our entire material of 620 individu-

als (1240 alleles) suggest that the *NURR1* gene does constitute an interesting gene for mutation screening in diseases with an involvement of the dopamine system.

Figure 3

Table 1

Fragment number	Description	Primer sequences
0	Part of the second (noncoding) exon, intron 1 (complete) and 2 bases of exon 3	F – GGAGATTGGACAGGCTGGAC R - TGCGCCTGAACACAAGGCAT
1	The forward primer is located in the second intron, immediately adjacent to the border to the third exon. This fragment contains the first 513 bases of exon 3	F – TTATCACCCTGTTTCATTTCC R - GAGACTGGCGTTTTCCTCT
2	Second half of exon 3. Both primers are located within coding sequence	F – TGCCGCACTCCGGGTCGGTTTACTACA R - GCCCTCACAGGTGCGCACGCCGTA
3	Rest of exon 3, complete intron 3 and major part of exon 4	F – CACGCGTCTCAGCTGCTCGACAC R - CTTCTTTGACCATCCCAACAGCCA
4	Exon 4, intron 4 and exon 5	F – CGCACAGTGCAAAAAAATGCAA R – CCTGGAATAGTCCAGGCTGG
5	Exon 5, intron 5 and exon 6	F – TGGTTCGCACAGACAGTTTA R - GCTAATCGAAGGACAAACAG
6	Exon 6, intron 6 and exon 7	F – TTCCAGGCGAACCCTGACTA R - ACCATAGCCAGGGCAGCAAT
7	Exon 7, intron 7 and exon 8 including 34 bases of 3' untranslated region	F – TCCAACCCAGTGGAGGGTAA R - ATTCCAGTTCCTTTGAAGTGC

Figure 4

Table 2

Mutation number	Diagnosis	Country of origin	Age of onset	Prominent symptoms	Affected family members
M1	Schizophrenia	Sweden	26	Auditory hallucinations, delusions of reference, and paranoid delusions.	Paternal grandmother's sister
M2	Bipolar disorder with psychotic features	Sweden	21	Auditory hallucinations, delusions of reference, incoherence and depressed mood.	Paternal grandfather
M3	MD (past); Childhood-onset schizophrenia, SAP, ADHD (current)	USA	6	Extended depressed mood, anhedonia, auditory hallucinations and delusions.	Not known

AMENDED CLAIMS

[received by the International Bureau on 24 November 2000 (24.11.00);
original claims 17 and 22 amended;
remaining claims unchanged (3 pages)]

1. An isolated Nurr1 gene including one or more mutations selected from the group consisting of Met97Val (M97V), His103Arg (H103R), Tyr121del (Y121del) and Tyr122del (Y122del), or a functional fragment or variant thereof.
2. A fragment according to claim 1, which comprises the exons of the Nurr1 gene.
3. A fragment according to claim 1 or 2, which comprises exon 3 of the Nurr1 gene.
4. A fragment according to any one of claims 1-3, which comprises the mutation Met97Val.
5. A fragment according to any one of claims 1-3, which comprises the mutation His103Arg.
6. A fragment according to any one of claims 1-3, which comprises the mutation Tyr121del or Tyr122del.
7. A nucleic acid capable of specifically hybridising to a gene or a fragment according to any one of claims 1-6.
8. A vector comprising a nucleic acid according to any one of claim 1-7.
9. A recombinant cell comprising a vector according to claim 8.
10. An isolated cell carrying one or more mutations selected from the group consisting of Met97Val (M97V), His103Arg (H103R), Tyr121del (Y121del) and Tyr122del (Y122del) in its genome.
11. A cell culture comprising cells according to claim 9 or 10, which cells are immortalized cells, such as mammalian cells.
12. A protein or a peptide encoded by a gene or a gene fragment according to any one of claims 1-7.
13. A protein or peptide according to claim 12, which includes a Val residue in the position corresponding to amino acid no. 97 of the wild type Nurr1 protein.
14. A protein or peptide according to claim 12, which includes an Arg residue in the position corresponding to amino acid no. 103 of the wild type Nurr1 protein.
15. A protein or peptide according to claim 12, which does not include any Tyr residue in the position corresponding to amino acid no. 121 or 122 of the wild type Nurr1 protein.

16. A method of screening for pharmaceutically active substances, wherein a nucleotide according to any one of claims 1-7 or a protein or peptide according to any one of claims 12-15 is used as a lead compound to identify substances capable of altering the biological effect of said nucleotide, or protein or peptide.
17. A method for the production of a pharmaceutical composition comprising the method according to claim 16 and furthermore mixing the substance identified with a pharmaceutically acceptable carrier.
18. An antibody raised against a protein or peptide according to any one of claims 12-15.
19. A transgenic, non-human animal, such as a mouse or a rat, comprising a gene or a gene fragment according to any one of claims 1-7.
20. A transgenic mouse comprising a mutation in the chromosome corresponding to the human chromosome 2q22-23 of said mouse, or an ancestor thereof, introduced at an embryonic stage such that said transgene replaces an endogenous allelle resulting in said mutation, which transgenic mouse comprises one or more mutations selected from the group consisting of Met97Val (M97V), His103Arg (H103R), Tyr121del (Y121del) and Tyr122del (Y122del).
21. A method of screening for pharmaceutically active substances, wherein an animal according to claim 19 or 20 is used as a lead to identify substances capable of altering the biological effect of a nucleotide according to any one of claims 1-7 and/or a protein or peptide according to any one of claims 12-15.
22. A method for the production of a pharmaceutical composition comprising the method according to claim 21 and furthermore mixing the substance identified with a pharmaceutically acceptable carrier.
23. An antibody according to claim 18 or a substance according to claim 17 or 22 for use as a medicament.
24. Use of an antibody according to claim 18 or a substance according to claim 17 or 22 in the manufacture of a medicament for the treatment and/or prevention of psychotic conditions, such as schizophrenia and/or manic depressive disorder.

25. A pharmaceutical preparation comprising an antibody according to claim 18 or a substance according to claim 17 or 22 together with a pharmaceutically acceptable carrier.

26. A method of detecting the presence of a mutation in exon 3 of the Nurr1 gene, which mutation is selected from the group consisting of Met97Val, His103Arg, Tyr121del and Tyr122del, said method comprising obtaining a biological sample from a mammalian, such as a human, subject and analyzing said sample for said mutation.

27. A method according to claim 26, wherein the biological sample is analyzed by isolating DNA from said sample, amplifying said DNA, hybridising said DNA to a labeled oligonucleotide probe that specifically hybridizes to mutant DNA containing a G as the first base of codon no 97; a G as the second base of codon no 103; or a deleted TAC in codon no 121 or 122, or to the close vicinity of said DNA.

28. A kit for performing the method according to claim 26 or 27, which kit comprises:

(a) reagents for amplification of one or more of the mutated sites; and/or

(b) enzymes for specific cleavage of DNA; and

(c) optionally suitable labels.

29. A method of treating and/or preventing a condition associated with schizophrenia and/or manic depression in a patient in need of therapy, wherein a mutation in exon 3 of the Nurr1 gene is corrected, which mutation is selected from the group consisting of Met97Val, His103Arg, Tyr121del and Tyr122del.

30. A method according to claim 29, wherein the DNA of one or more of said mutations is replaced by DNA having the native, non-mutated base sequence using a vector suitable for transfecting the patient.

31. A method according to claim 29, wherein cells comprising the native, non-mutated base sequence in the positions corresponding to one or more of said mutations are introduced in said patient.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

REVISED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 January 2001 (04.01.2001)

PCT

(10) International Publication Number
WO 01/00807 A1

(51) International Patent Classification[7]: C12N 15/00, C07K 14/705, A61K 39/395, A01K 67/027

(21) International Application Number: PCT/SE00/01380

(22) International Filing Date: 29 June 2000 (29.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9902489-5 30 June 1999 (30.06.1999) SE

(71) Applicant *(for all designated States except US)*: **KAROLINSKA INNOVATIONS AB** [SE/SE]; S-171 77 Stockholm (SE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **BUERVENICH, Silvia** [DE/SE]; Berga Backe 6, S-182 53 Danderyd (SE). **OLSON, Lars** [SE/SE]; Ankarvägen 1, S-181 43 Lidingö (SE). **ANVRET, Maria** [SE/SE]; Statyvägen 4, S-175 47 Järfälla (SE). **CARMINE, Andrea** [SE/SE]; Byggmästarvägen 6, S-168 32 Bromma (SE).

(74) Agent: **KILANDER, Annika**; Göteborgs Patentbyrå Dahls AB, P.O. Box 606, S-182 16 Danderyd (SE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *With amended claims.*

(88) Date of publication of the revised international search report: 19 April 2001

(15) Information about Correction:
see PCT Gazette No. 16/2001 of 19 April 2001, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/00807 A1

(54) Title: MUTATED Nurr1 GENE

(57) Abstract: The present invention relates to an isolated Nurr1 gene including one or more mutations selected from the group consisting of Met97Val (M97V), His103Arg (H103R), Tyr121del (Y121del) and Tyr122del (Y122del), or a functional fragment or variant thereof, as well as to proteins or peptides encoded thereof. Further, the invention also relates to cell cultures and transgenic animals comprising a mutated gene or a gene fragment as models for the study of psychotic disorders, such as schizophrenia and/or manic depressive disorder, as well as for the identification of effective therapies and drugs for the treatment of said disorders. In an additional aspect the invention relates to novel drugs developed by use of one or more of the mutations according to the invention for the treatment and/or prevention of psychotic disorders. Finally, the invention relates to methods of diagnostis wherein the mutations according to the invention are identified as well as to kits for performing such methods.

Figure 1

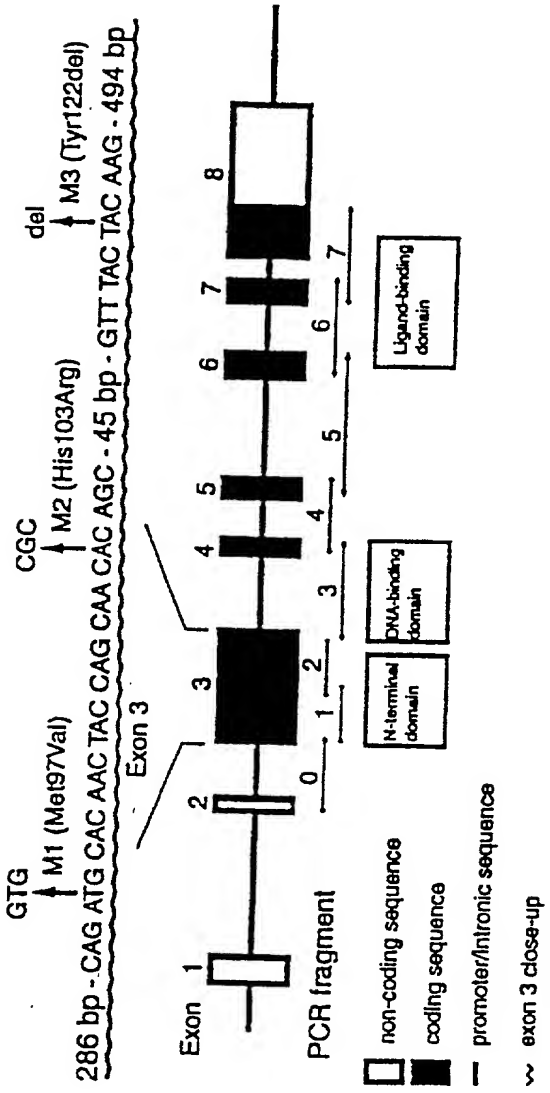


Figure 1

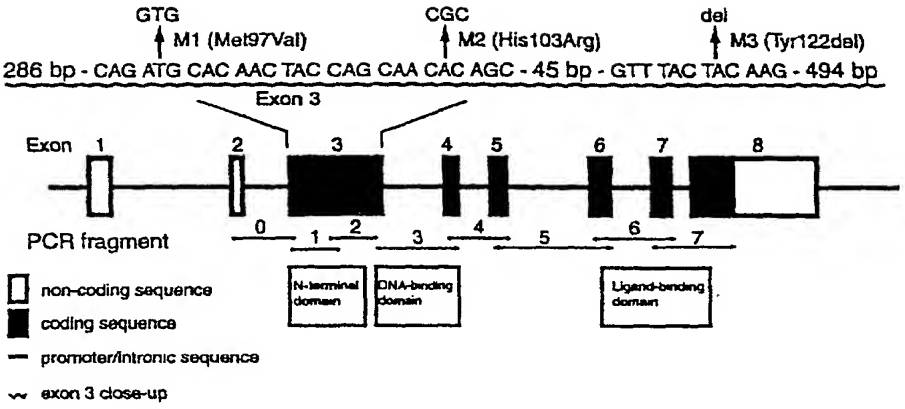
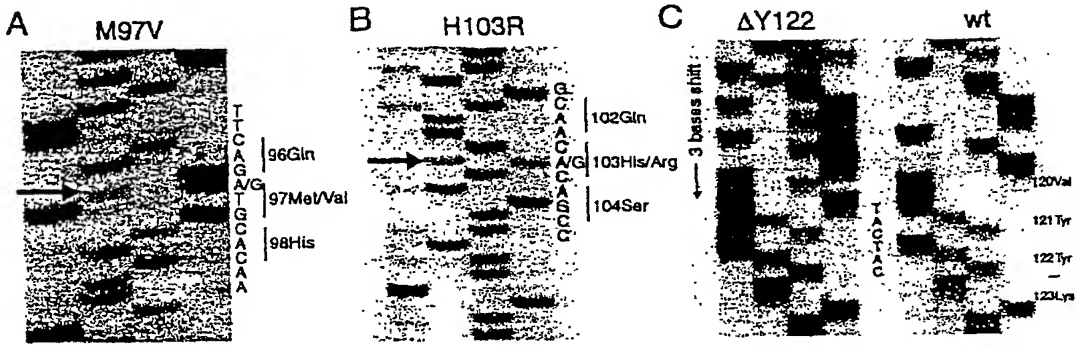


Figure 2



cover the fee under 37 C.F.R. §1.492(e) for providing a declaration after the filing date. As the Declaration is being filed within two months of the mailing date of the Notice, an extension of time is not required.

Please note that during the interval between preparation of the Declaration for signature and the execution of the Declaration by the inventors, the residential address for inventor Silvia Buervenich changed. Accordingly, inventor Buervenich crossed-out her previous address, as provided in the prepared Declaration, and printed her current address in the appropriate spaces prior to signing the Declaration. Her new address is: Tegnérgatan 55, S-11161, Stockholm, Sweden.

If any additional fee is required with this communication, authorization is hereby given to charge any such fee to Deposit Account No. 23-1703.

Dated: April 18, 2002

Respectfully submitted,

Andrew Fessak

Andrew Fessak
Reg. No. 48,528
Agent for Applicants

Customer No. 007470
(212) 819-8437

Enclosure

05/01/2002 MAL111 00000024 231703 10019365
01 FC:154 130.00 CH

AZ ref: 100587-1P US

Docket Number:

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to our names.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

MUTATED Nurr1 GENE,

the specification of which is attached hereto unless the following box is checked:

☒ was filed on 29 June 2000 as United States Application Number _____ or PCT International Application Number PCT/SE00/01380 and was amended on _____(if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
9902489-5	Sweden	30 June 1999	☐
Number	Country	Day/Month/Year Filed	
			☐
Number	Country	Day/Month/Year Filed	

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

(Application Number) (Filing Date)

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

(Application Number) (Filing Date) (Status--patented, pending, abandoned)

(Application Number) (Filing Date) (Status--patented, pending, abandoned)

I/we hereby appoint **Practitioners at Customer Number 007470** as my/our attorney(s) or agent(s) to prosecute the application identified above, and to transact all business in the United States Patent and Trademark Office connected therewith.

Address all correspondence to **Customer No. 007470**
Telephone No.: 212-819-8200;
Facsimile No.: 212-354-8113

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believe to be true; and further that these statements were made with
the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor (first name, middle initial, last name):
Silvia Buervenich

First inventor's signature Silvia Buervenich Date: 10/4-02

Residence: ~~Danderyd~~, Sweden Citizenship: German
Post Office Address: ~~Berga Backe 6, S-182 53 Danderyd~~, Sweden
Tegnérgatan 55, S-11161 Stockholm

Full name of second inventor (first name, middle initial, last name):
Lars Olson

Second inventor's signature Lars Olson Date: 10/4-02

Residence: Lidingö, Sweden Citizenship: Swedish
Post Office Address: Ankarvägen 1, S-181 43 Lidingö, Sweden

Full name of third inventor (first name, middle initial, last name):
Maria Anvret

Third inventor's signature Maria Anvret Date: 4/2-02

Residence: Järfälla, Sweden Citizenship: Swedish
Post Office Address: Statyvägen 4, S-175 47 Järfälla, Sweden

Full name of fourth inventor (first name, middle initial, last name):
Andrea Carmine

Fourth inventor's signature Andrea Carmine Date: 10/4-02

Residence: Bromma, Sweden Citizenship: Swedish
Post Office Address: Byggmästarvägen 6, S-168 32 Bromma, Sweden